AD_____

Award Number: DAMD17-00-1-0403

TITLE: Impact of Disrupted BRCA2 Protein-Protein Interactions on DNA Repair and Tumorigenesis

PRINCIPAL INVESTIGATOR: Christopher J. Sarkisian
Lewis A. Chodosh, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual Summary (01 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Impact of Disrupted BRCA2 Protein-Protein Interactions on DNA Repair and Tumorigenesis

5. FUNDING NUMBERS
DAMD17-00-1-0403

6. AUTHOR(S)
Christopher J. Sarkisian
Lewis A. Chodosh, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-Mail: csarkisl@mail.med.upenn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

In this report, we have analyzed the protein encoded by the murine *Brca2* locus. We find that murine Brca2 shares multiple properties with human BRCA2 including its regulation during the cell cycle, localization to nuclear foci, and interaction with Brca1 and Rad51. Murine Brca2 stably interacts with human BRCA1, and the amino-terminus of Brca2 is sufficient for this interaction. Exon 11 of murine Brca2 is required for its stable association with RAD51, whereas the carboxyl-terminus of Brca2 is dispensable for this interaction. Finally, in contrast to human BRCA2, we demonstrate that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. This finding may explain the apparent inconsistency between the cytoplasmic localization of carboxyl-terminal truncations of human BRCA2 and the hypomorphic phenotype of mice homozygous for similar carboxyl-terminal truncating mutations.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

___ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

12.20.01

PI - Signature Date

Table of Contents

INTRODUCTION

Breast cancer arises through multiple mutations in a single mammary epithelial cell, particularly those involving tumor suppressor genes, proto-oncogenes, and genes involved in DNA repair. The most rational approach to the treatment of breast cancer would therefore rely on knowledge of the particular molecules mutated or deleted in each clonogenic tumor. One of the molecules most commonly mutated in hereditary breast cancer is the tumor suppressor gene, *BRCA2*. *BRCA2* is mutated in approximately 35% of all multiple case, early-onset hereditary female breast cancers as well as in a significant portion of hereditary male breast cancers. These tumors frequently occur with a higher grade and larger degree of aneuploidy than sporadic tumors. Recent evidence has suggested that *BRCA2* may play a role in regulating genomic integrity of cells during proliferation and following DNA damage. Intriguingly, the properties of the familial breast cancer tumor suppressor, *BRCA1*, in spite of its lack of homology to *BRCA2*, have been demonstrated to be similar to those of *BRCA2* in almost every other respect. Moreover, BRCA1 has been shown to physically interact with BRCA2 *in vivo*. Due to the striking convergence of these two different breast cancer predisposition genes, we hypothesize that the association of BRCA2 with BRCA1 comprises a critical part of BRCA2's role as a genomic caretaker and breast tumor suppressor. In this proposal we will determine if this hypothesis is correct by developing an animal model in which BRCA2 is deficient in its ability to interact with BRCA1. Specifically, we will: 1) Determine whether Brca1 and Brca2 interact in murine cells; 2) Map the domain(s) of Brca2 necessary for such an interacting with Brca1; and 3) Determine whether mice bearing a *Brca2* allele defective in binding to Brca1 are genomically unstable or susceptible to tumorigenesis.

BODY

The goals of my 1999 pre-doctoral fellowship "Role Of *Brca2* In Cell Cycle Progression And The Response To DNA Damage In Mammary Epithelial Cells", were to 1) Verify and Map the Brca2 and Brca1 Interaction *In Vivo* and 2) Determine if the Brca2 interaction with Brca1 is necessary for DNA Repair and Tumor Suppression *In Vivo*. We have published results addressing aim 1 in the manuscript, "Analysis Of Murine Brca2 Reveals Conservation Of Protein-Protein Interactions But Differences In Nuclear Localization Signals" (Appendix 1) (1). In this manuscript we have addressed aim 1 by verifying the stable interaction between murine Brca2 and Brca1 (please see attached manuscript for details, Fig 4) and by demonstrating that the amino-terminus of murine Brca2 is sufficient for interaction with human BRCA1 (please see attached manuscript for details, Fig 6). Also in this manuscript, we have characterized the murine Brca2 protein. We find that Brca2 stably interacts with murine Rad51 (please see attached manuscript for details, Fig 4). We demonstrate that the physical association of Brca2 with Rad51 requires exon 11 of murine Brca2, but not its carboxyl-terminus (please see attached manuscript for details, Fig 5). We also show that murine Brca2 differs from human BRCA2 in that carboxyl-terminal truncations of murine Brca2 localize to the nucleus (please see attached manuscript for details, Fig 7). Collectively, our findings suggest that multiple functional interactions of Brca2 have been evolutionarily conserved with the notable exception of those signals required for its nuclear localization.

To further define the region of murine Brca2 required for interaction with Brca1, we have generated an amino-terminal *Haemagglutinin* (HA)-tagged full-length murine *Brca2* cDNA. Using this HA-tagged full-length *Brca2* cDNA, we have also cloned three *Brca2* mutants containing in-frame deletions of the amino terminus, exon 11, and the carboxyl-terminus of Brca2, respectively. We will determine regions of Brca2 required for interaction with Brca1 by transfecting in the HA-tagged deletion constructs, performing anti-HA immunoprecipitations, and immunoblotting for co-precipitating Brca1. We have also cloned 19 overlapping GST-Brca2 fusion polypeptides, each containing approximately 230 amino acids of Brca2 sequence, collectively spanning the entire Brca2 protein. We will perform GST pulldowns from mammary epithelial cell

line protein extracts and immunoblot for co-precipitating Brca1 to more precisely identify regions of Brca2 critical for interaction with Brca1.

KEY RESEARCH ACCOMPLISHMENTS

- Murine Brca2 interacts with murine and human BRCA1 *in vivo*
- The amino-terminus of murine Brca2 is sufficient to interact with human BRCA1
- Murine Brca2 protein levels are cell cycle regulated such that it is upregulated at the G1/S cell cycle boundary
- Murine Brca2 is nuclear and localizes to subnuclear foci in mammary epithelial cell lines
- The majority of murine Brca2 protein is stably associated with Rad51 *in vivo*
- Exon 11 of murine Brca2 is required for association with Rad51, and the carboxyl-terminus of Brca2 is dispensable for this interaction
- Unlike human *BRCA2*, carboxyl-terminal truncating mutations of *Brca2* generate polypeptides that retain nuclear localization

REPORTABLE OUTCOMES

Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) J. Biol. Chem. **276**, 37640-37648

CONCLUSIONS

We have shown that the interaction between murine Brca2 and Brca1 exists in vivo. Furthermore, we have shown that the amino terminus of murine Brca2 is sufficient to interact with human BRCA1. We have generated reagents such as epitope tagged murine Brca2 deletion constructs and GST fusion proteins to more completely map this interaction.

We have also generated other new information regarding murine Brca2. We have demonstrated that the murine Brca2 protein is similar to human BRCA2 with regard to its nuclear localization, cell cycle regulation, binding to Brca1, and binding to Rad51. In addition, we shown that exon 11 of Brca2 is required for its interaction with RAD51. Finally, despite low overall homologies between the murine and human orthologs of BRCA2 and BRCA1, we have demonstrated that murine Brca2 is capable of stably interacting with human BRCA1 *in vivo*. This indicates that the interaction between Brca1 and Brca2 has been conserved evolutionarily and suggests that this interaction is functionally important. In aggregate, our data are consistent with the hypothesis that murine and human BRCA2 have largely equivalent functions.

Future experiments will include the generation of a Brca2 targeting construct that contains a Brca2 mutant lacking the Brca1 binding domain. Embryonic stem cells will be electroporated with this construct, screened to verify that a correct recombination event has occurred, and injected into blastocysts for the generation of Brca2 knock-in mice. We will determine that these mice lack a stable association between murine Brca2 and Brca1, and will determine their susceptibility to spontaneous and induced tumorigenesis, as well as genotoxic insult. These experiments will determine the significance of the Brca1-Brca2 interaction in the role of Brca2 as a tumor suppressor and guardian of the genome.

REFERENCES

1. Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) J. Biol. Chem. **276**, 37640-37648

APPENDICES

Appendix 1 (Attached) - Sarkisian, C.J., Master, S.R., Huber, L.J., Ha, S.I., and Chodosh, L.A. Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals. (2001) J. Biol. Chem. **276**, 37640-37648

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 40, Issue of October 5, pp. 37640–37648, 2001
Printed in U.S.A.

Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals*

Received for publication, July 5, 2001, and in revised form, July 23, 2001
Published, JBC Papers in Press, July 26, 2001, DOI 10.1074/jbc.M106281200

Christopher J. Sarkisian‡, Stephen R. Master‡, L. Julie Huber‡, Seung I. Ha‡ and Lewis A. Chodosh‡§¶

From the ‡Department of Molecular and Cellular Engineering and §Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6160

In this report, we have analyzed the protein encoded by the murine *Brca2* locus. We find that murine Brca2 shares multiple properties with human BRCA2 including its regulation during the cell cycle, localization to nuclear foci, and interaction with Brca1 and Rad51. Murine Brca2 stably interacts with human BRCA1, and the amino terminus of Brca2 is sufficient for this interaction. Exon 11 of murine Brca2 is required for its stable association with RAD51, whereas the carboxyl terminus of Brca2 is dispensable for this interaction. Finally, in contrast to human BRCA2, we demonstrate that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. This finding may explain the apparent inconsistency between the cytoplasmic localization of carboxyl-terminal truncations of human BRCA2 and the hypomorphic phenotype of mice homozygous for similar carboxyl-terminal truncating mutations.

Women inheriting mutations in the *BRCA2* tumor-suppressor gene have up to an 84% lifetime risk of developing breast cancer (1), and these tumors account for ~35% of inherited breast cancers in women (2). *BRCA2* encodes a 3418-amino acid nuclear protein of a predicted molecular mass of 384 kDa. Most disease-causing *BRCA2* alleles contain truncating mutations that result in deletion of the three characterized nuclear localization signals present at the extreme carboxyl terminus of BRCA2 (3, 4). Because these signals are required for the nuclear localization of human BRCA2, it has been postulated that truncating alleles of *BRCA2* are functionally equivalent to null alleles of this tumor suppressor gene (3).

Though its exact cellular role remains unclear, a growing body of evidence indicates that BRCA2 is involved in DNA damage-response pathways shared with BRCA1 and RAD51. BRCA2, BRCA1, and RAD51 are each co-regulated with highest levels of expression occurring during the S and G_2/M phases of the cell cycle, and these proteins co-localize to discrete foci within the nucleus (5–11). Furthermore, human BRCA2 has been shown to physically interact with both RAD51 (12–16) and BRCA1 (16).

Human BRCA2 binds to RAD51 via eight BRC repeats, each 30–80 amino acids in length, that are located within exon 11 of BRCA2 (17, 18). These repeats have been demonstrated by yeast two-hybrid analysis to be both necessary and sufficient for stable binding of human BRCA2 to RAD51 (12, 14, 15). The region(s) of BRCA2 that are required for binding to BRCA1 have been less clearly defined, though the carboxyl-terminal third of BRCA2 has been shown to be dispensable for this interaction (16). Nevertheless, despite the identification of BRCA2-RAD51 and BRCA2-BRCA1 protein-protein interactions, the contribution of these interactions to the tumor-suppressor functions of BRCA2 remains uncertain.

Mice bearing homozygous mutations in *Brca2* that yield truncations of all eight BRC repeats uniformly die *in utero* between embryonic day 6.5-8.5, with elevated levels of p53 and p21 (19–21). Notably, this phenotype is similar to that of mice homozygous for null mutations in either *Rad51* or *Brca1* (22–26). Whereas mice bearing truncating alleles of *Brca2* that remove only a subset of BRC repeats also die *in utero*, a fraction of homozygotes survive to birth with the survival rate being roughly proportional to the number of BRC repeats left intact (27–29). Surviving homozygotes invariably succumb to thymic lymphomas, and cells from these mice exhibit increased genotoxin sensitivity and chromosomal instability, as well as an impaired ability to form Rad51 nuclear foci in response to DNA damage (27–30). In contrast, mice homozygous for truncating mutations in *Brca2* that leave exon 11 intact exhibit a more limited sensitivity to genotoxins, are 100% viable, and do not appear to develop spontaneous tumors (31). These data argue for a central role of exon 11 in the genomic surveillance and tumor-suppressor functions of Brca2.

Whereas murine knockout models support a role for BRCA2 as a tumor suppressor, the increasingly severe defects observed in mice as larger amounts of the Brca2 carboxyl terminus are truncated appear inconsistent with reports that even small carboxyl-terminal truncations in human BRCA2 result in its cytoplasmic localization. That is, essentially all truncating alleles might be expected to behave similarly to null alleles, because carboxyl-terminal truncation would ostensibly lead to cytoplasmic localization and preclude Brca2 from participating in nuclear functions (3, 4). This apparent discrepancy could be because of differences in the subcellular localization signals of human and murine Brca2 or to differences in the functions of murine and human BRCA2 in the cytoplasm. In this regard, another apparent functional difference between murine and human BRCA2 is suggested by the mapping of a murine Brca2-Rad51 interaction to the carboxyl terminus of murine Brca2, because similar approaches have shown that the corresponding region of human BRCA2 lacks significant affinity for RAD51 (12, 14, 15). Further complicating the direct comparison of

* This work was supported in part by NCI, National Institutes of Health Grants CA71513 and CA78410 and by United States Army Breast Cancer Research Program Grants DAMD17-00-1-0403 (to C. J. S.), DAMD17-98-1-8230 (to L. J. H.), and DAMD17-98-1-8226 (to L. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Molecular and Cellular Engineering, 612 Biomedical Research Building II/III, University of Pennsylvania School of Medicine, 421 Curie Blvd., Philadelphia, PA 19104–6160. Tel.: 215-898-1321; Fax: 215-573-6725; E-mail: chodosh@mail.med.upenn.edu.

murine and human BRCA2 is the fact that the overall amino acid homology between these orthologs is only 59%, a relatively low degree of evolutionary conservation compared with other tumor-suppressor genes. Together, these data have called into question the applicability of murine models for understanding the function of human BRCA2.

In this report, we characterize the murine Brca2 protein. We find that Brca2 stably interacts with murine Brca1 and Rad51. We demonstrate that the physical association of Brca2 with Rad51 requires exon 11 of murine Brca2 but not its carboxyl terminus. We also show that murine Brca2 differs from human BRCA2 in that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. Collectively, our findings suggest that multiple functional interactions of Brca2 have been evolutionarily conserved with the notable exception of those signals required for its nuclear localization.

EXPERIMENTAL PROCEDURES

Isolation of Murine Brca2 cDNA—Poly(A)$^+$ RNA isolated from day 14 murine embryos was used to generate a cDNA library in lambda ZAP using the ZAP-cDNA synthesis and ZAP-cDNA Gigapack II Gold packaging kits according to manufacturer's instructions (Stratagene). 5×10^5 plaques from each library were screened by standard methods using [^{32}P]dCTP-labeled random-primed cDNA fragments (BMB) corresponding to nucleotides 2–221, 798–2932, and 9033–9972 of murine *Brca2*. Hybridization was performed at a concentration of 10^6 cpm/ml in 48% formamide, 10% dextran sulfate, 4.8× SSC, 20 mM Tris, pH 7.5, 10× Denhardt's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42 °C overnight. Filters were washed twice in 2× SSC/0.1% SDS at room temperature for 20 min and twice in 0.2× SSC/0.1% SDS for 20 min at 50 °C and subjected to autoradiography on XAR-5 film (Eastman Kodak Co.). Phage clones were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions. Sequence analysis identified three overlapping clones that together spanned the entire *Brca2* coding sequence, with the exception of an internal deletion of nucleotides 454–672. This region was replaced with a polymerase chain reaction product generated from murine testis first-strand cDNA and primers 5′-GAATTCATGCCCGTTGAATACAAAAGGAGAC-3′ and 5′-CTCGAGGCAGATTTCCTCATTCTGGCTG-3′. After sequencing to verify the absence of additional mutations, the overlapping clones were assembled to generate a full-length murine *Brca2* cDNA.

Generation of Antisera—Using primers 5′-CATCCGAATTCTGCAGCACAGCGATTTAGGAC-3′ and 5′-CATCCCTCGAGGCACCGCAGAGTAAGAGGG-3′ (Brca2A), and 5′-CATCCGAATTCGATGAAGAAGCACGCAGCTC-3′ and 5′-CATCCCTCGAGACTGCATTTTTCACAGTGGC-3′ (Brca2B), polymerase chain reaction products corresponding to amino acids 19 to 135 (Brca2A) and 206 to 566 (Brca2B) were generated from a partial *Brca2* cDNA, ligated into pGEM-T vector (Promega), and subcloned in-frame into pGEX-6P-1 (Amersham Pharmacia Biotech). GST[1] fusion peptides were purified from BL21 *Escherichia coli* according to manufacturer's instructions. Brca2 peptides were cleaved from the GST domain using a site-specific protease, gel-purified by SDS-PAGE, and injected into rabbits using standard immunization protocols (Cocalico Biologicals). Sera from immunized rabbits were affinity-purified on columns containing immunogen bound to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) according to published methods (32).

Transfection of Cells—293T cells were transiently transfected using a standard calcium-phosphate protocol (33). For co-immunoprecipitation experiments, 2.5×10^6 cells on 150-mm dishes were transfected with 25 μg of DNA. For subcellular localization studies, 1×10^6 cells on 100-mm dishes were transfected with 5 μg DNA, and cells were split onto culture slides at 24 h post-transfection. All analyses were performed at 48 h post-transfection.

Cell Culture—All cells were grown at 37 °C in a humidified incubator supplemented with 5% CO_2. 293T, NMuMG, and 16MB9A cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (Gem Cell), 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin. HC11 cells were cultured in RPMI supplemented with 10% bovine calf serum (Gem Cell), 1 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, 10 ng/ml epidermal growth factor, and 5 μg/ml insulin.

Immunoblotting and Immunoprecipitation—Cells were harvested by lysis in EBC Buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Igepal CA-630 (Sigma)), supplemented with phosphatase inhibitors (50 mM NaF and 1 mM β-glycerol phosphate) and protease inhibitors (100 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pefabloc). Following removal of insoluble debris by centrifugation, extracts were either boiled in 1× (final) Laemmli sample buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8, 5% β-mercaptoethanol, 250 mM dithiothreitol, 0.005% bromphenol blue) or subjected to immunoprecipitation. For immunoprecipitation studies, 1.0–1.5 mg of protein extract was incubated with 4 μg of affinity-purified antibody or, in the case of RAD51, 1 μl of polyclonal antiserum (Ab-1; Oncogene Science) for 1 h at 4 °C in a total volume of 1 ml of EBC plus inhibitors. Protein A-Sepharose (25 μl of a 50% slurry in PBS; Life Technologies, Inc.) was added, and incubation was continued for 1 h. The Sepharose beads were pelleted and washed three times in EBC plus inhibitors, resuspended, boiled in 14 μl of 2× (final) Laemmli sample buffer, and loaded for SDS-PAGE.

Except as noted, all protein samples were separated by 5% SDS-PAGE in 50 mM Tris base, 192 mM glycine, and 0.1% SDS. For immunoblotting, electrophoresed proteins were transferred onto nitrocellulose (Schleicher & Schuell) in 50 mM Tris base, 192 mM glycine, and 20% methanol in a submerged tank apparatus (Bio-Rad) for 12 h at 20 V. Blotted membranes were rinsed twice in PBS and blocked for 1 h at room temperature in PBS containing 5% nonfat milk and 0.05% Igepal CA-630 (MPBS-I). All affinity-purified rabbit polyclonal primary antibodies were used for immunoblotting at a final concentration of 2 μg/ml. Commercial antibodies, including anti-human BRCA2 Ab-2 (Oncogene Science), anti-RAD51 Ab-1 (Oncogene Science), anti-RAD51 Ab-1 (NeoMarkers), anti-BRCA1 MS110 (Oncogene Science), anti-β-tubulin N357 (Amersham Pharmacia Biotech), and anti-RAD50 R75020 (Transduction Laboratories) were used at the concentrations recommended by the manufacturer. Blots were incubated with primary antibodies diluted in MPBS-I for 1 h at room temperature and were subsequently washed three times in MPBS-I for 10 min each. Peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson Immunoresearch) were incubated at a 1:5000 dilution for 1 h at room temperature in MPBS-I. Blots were washed three times in MPBS-I for 15 min each and rinsed four times with PBS, and antibody complexes were detected by chemiluminescence (ECL Plus; Amersham Pharmacia Biotech) on XAR-5 film (Kodak).

Cell Cycle Synchronization and Analysis—HC11 cells were synchronized by serum starvation for 48 h and were subsequently restimulated with growth medium containing 20% serum. At 4-h intervals, cells were trypsinized and washed in PBS, and approximately two-thirds of cells were pelleted and snap-frozen for subsequent protein harvest. The remaining cells were pelleted, resuspended in PBS, and fixed in 70% ethanol. Following fixation, cells were pelleted, resuspended in PBS supplemented with 10 μg/ml propidium iodide and 100 μg/ml RNase A, and sorted by DNA content using a Becton Dickinson FACScan flow cytometer. The program ModFit was used to quantify percentages of cells in each phase of the cell cycle.

Subcellular Fractionation—Nuclear and cytoplasmic fractionation was performed as described previously (34). Briefly, 16MB9A cells were harvested by trypsinization, pelleted, and washed in PBS. Cells were washed in ice-cold hypotonic buffer (30 mM HEPES, pH 7.5, 5 mM KCl, 1 mM $MgCl_2$), resuspended in three packed cellular volumes of hypotonic buffer supplemented with protease inhibitors, and incubated on ice for 30 min. Cells were homogenized in a Wheaton Dounce with 25 strokes of a type B pestle. An equal volume of Nonidet P-40 lysis buffer (0.1% Igepal CA-630, 250 mM sucrose, 1 mM $MgCl_2$, 10 mM Tris, pH 7.5) was added dropwise, and cells were lysed using another 10 strokes. Nuclei were pelleted at 1300 × *g* at 4 °C for 5 min. Following removal of the cytoplasmic supernatant, nuclei were washed twice in 1:1 hypotonic buffer/Nonidet P-40 lysis buffer and resuspended in an amount of 1:1 hypotonic buffer/Nonidet P-40 lysis buffer equal to the extract volume prior to centrifugation of nuclei. Nuclear and cytoplasmic fractions were diluted with 6× EBC to a final concentration of 1×, centrifuged to remove insoluble debris, and boiled in 1× (final) Laemmli sample buffer prior to SDS-PAGE.

Immunofluorescence—Cells were cultured in 2-well culture slides (Falcon), rinsed in PBS, and fixed for 10 min in 3% paraformaldehyde/2% sucrose/PBS. Cells were rinsed twice in PBS and permeabilized for 5 min in ice-cold buffer (0.5% Triton, 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose). Following five rinses in PBS, cells were incubated at 37 °C for 20 min with anti-Brca2A (2 μg/ml in 3% bovine serum albumin/PBS). Cells were rinsed twice in PBS and

[1] The abbreviations used are: GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

To address this, we performed reciprocal co-immunoprecipitations of Rad51 and Brca2 from HC11 cells (Fig. 4*A*). This

(mAb-1) and residues 995–1244 (mAb-3) (36), we performed reciprocal co-immunoprecipitations of Brca1 with Brca2. Low

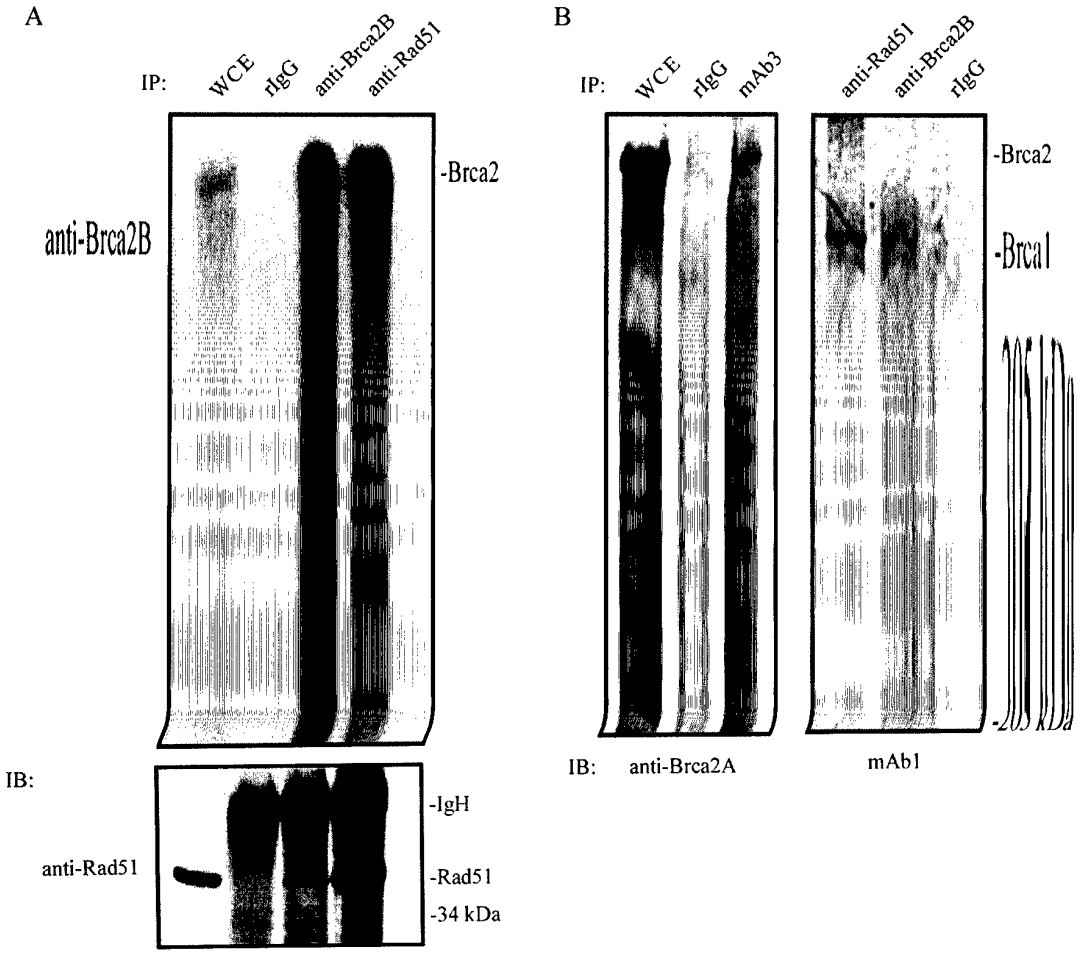

FIG. 4. **Brca2 interacts with Rad51 and with murine Brca1 *in vivo*.** *A*, asynchronously growing HC11 cells were harvested and immunoprecipitated using anti-Brca2B, anti-RAD51 Ab-1 (Oncogene Science), or rabbit IgG. Precipitates were separated by SDS-PAGE and immunoblotted with anti-Brca2A (*top panel*) or with anti-RAD51 Ab-1 (Oncogene Science; *bottom panel*). The whole cell extract (*WCE*) represents 1/30 of the input protein. *B*, Brca1 (*left panel*), Brca2 (*right panel*), or Rad51 (*right panel*) were immunoprecipitated (*IP*) from HC11 whole cell extracts using the indicated antibodies. Precipitates were immunoblotted (*IB*) with anti-Brca2A (*left panel*) or anti-Brca1 mAb-1 (*right panel*). The whole cell extract represents 1/20 of the input protein.

but significant levels of Brca2 were detected in Brca1 immunoprecipitates (Fig. 4*B*). Based on comparisons to input cellular extracts, less than 5% of total Brca2 polypeptides appear to be stably bound to Brca1 under these conditions (Fig. 4*B*). This finding is consistent with the fraction of total BRCA2 molecules that have been reported to be bound to BRCA1 in human cells (16). Conversely, we detected Brca1 in Brca2 immunoprecipitates at levels approximately equivalent to those present in Rad51 precipitates (Fig. 4*B*). No Brca1 or Brca2 was detected in rabbit IgG control precipitates. Taken together, our data indicate that the proteins with which murine Brca2 interacts are similar to those with which human BRCA2 interacts and that the stoichiometries with which these interactions occur may also be similar. Of note, however, we cannot rule out the possibility that our antibodies have incomplete access to cellular Brca2-Brca1 complexes or that the immunoprecipitation conditions employed in these experiments cause disruption of Brca2-Brca1 complexes.

Exon 11 of Murine Brca2, but Not Its Carboxyl Terminus, Is Required for Interaction with Rad51—The domain(s) with which murine and human BRCA2 each interact with RAD51 have been noted previously to differ (37). Specifically, murine Brca2 has been shown to interact with Rad51 via its carboxyl terminus by yeast two-hybrid and *in vitro* GST pulldown assays (19, 38). However, despite the high degree of evolutionary conservation of this domain, similar approaches have indicated that the corresponding domain of human BRCA2 does not

trol vector, and harvested cell extracts were subjected to immunoprecipitation for Brca2 and RAD51. This analysis revealed that full-length Brca2 and Brca2ΔC were comparable in their ability to co-precipitate RAD51 (Fig. 5*B*). In contrast, no RAD51 was detected in anti-Brca2 immunoprecipitates from cells transfected with *Brca2ΔBst* or *Brca2ΔBst-C*, despite the fact that these Brca2 mutant polypeptides were expressed at levels that exceeded those of full-length Brca2 and Brca2ΔC (Fig. 5*B*). Performing the reciprocal co-immunoprecipitation experiment in 293T cells yielded similar results, as full-length Brca2 and Brca2ΔC were found to co-immunoprecipitate with RAD51, whereas Brca2ΔBst and Brca2ΔBst-C failed to co-precipitate with RAD51 (Fig. 5*C*). These results were also observed when Brca2ΔBst and Brca2ΔBst-C were expressed at levels comparable with those of full-length Brca2 and Brca2ΔC (data not shown) indicating that the failure of Brca2ΔBst and Brca2ΔBst-C to interact with RAD51 is not an artifact of their higher expression levels. Moreover, the fact that human and murine RAD51 are identical at the amino acid level, and that the BRCA2-RAD51 interaction has been shown to be direct, suggests that the inability of Brca2ΔBst and Brca2ΔBst-C to co-precipitate RAD51 is not due to differences in RAD51 sequences or bridging molecules present in human cells (12, 14, 15). Collectively, these data strongly suggest that exon 11 is the principal RAD51 interaction domain contained within murine Brca2.

The Amino Terminus of Murine Brca2 Is Sufficient for Inter-

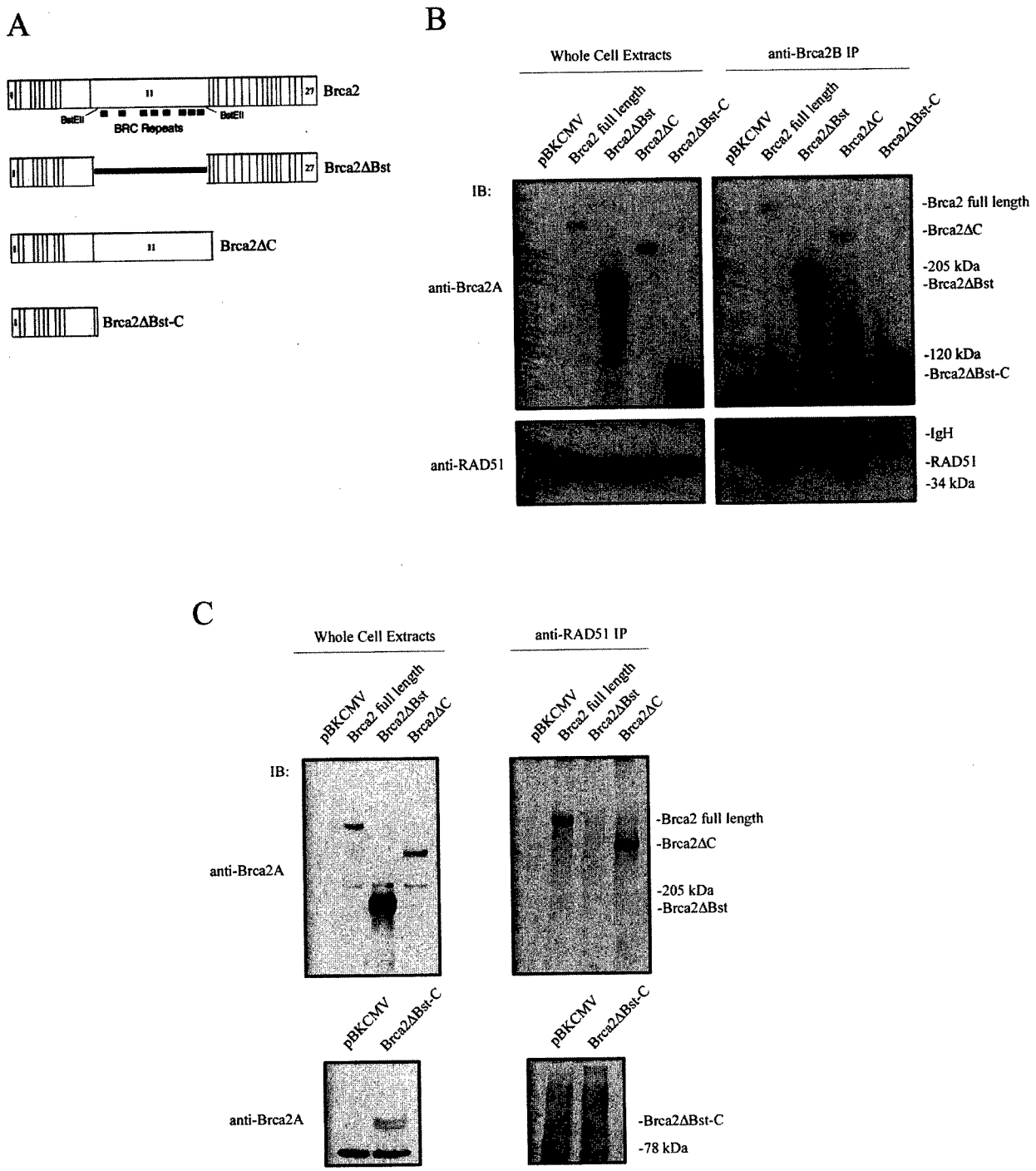

FIG. 5. **Exon 11, but not the carboxyl terminus, of murine Brca2 is required for interaction with RAD51.** *A*, schematic depicting deletion mutants of *Brca2* generated from a full-length *Brca2* cDNA. *Brca2ΔBst* represents an internal deletion mutant that encodes a polypeptide lacking amino acid residues 738–2278 from exon 11. *Brca2ΔC* lacks sequence from the second *Bst*EII site, at residue 2280, to the carboxyl terminus of the protein, and *Brca2ΔBst-C* lacks sequence from the first *Bst*EII site, at residue 742, to the carboxyl terminus of the protein. *B*, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Cellular extracts (*left panels*) or cellular extracts immunoprecipitated (*IP*) with anti-Brca2B (*right panels*), were separated by SDS-PAGE and immunoblotted with either anti-Brca2A (*top panel*) or anti-RAD51 Ab-1 (NeoMarkers; *bottom panel*). *C*, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Whole cell extracts (*left panels*) or whole cell extracts immunoprecipitated with anti-RAD51 Ab-1 (Oncogene Science; right panels) were immunoblotted (*IB*) with anti-Brca2A. The *bottom panels* were separated by 15 rather than 5% SDS-PAGE to enhance the resolution of Brca2ΔBst-C.

with the control vector, *pBKCMV*, suggesting that anti-Brca2A may cross-react, albeit weakly, with human BRCA2 (Fig. 6). In aggregate, our findings suggest that sequences within the amino-terminal 738 residues of Brca2 are sufficient for interaction with BRCA1, although additional domains of Brca2 may contribute to the stability of this association. These findings are consistent with experiments demonstrating that the interaction of human BRCA2 and BRCA1 is preserved in CAPAN-1 cells, which express a BRCA2 protein that lacks the carboxyl-terminal third of BRCA2 (16). To date, the lack of an antibody to the carboxyl terminus of Brca2 and inefficient expression of epitope-tagged Brca2 deletion mutants (data not shown) have prevented us from testing whether the amino terminus of Brca2 is required for its interaction with BRCA1. Nevertheless, we conclude that the amino terminus of murine Brca2 is sufficient to stably interact with human BRCA1 and that the se-

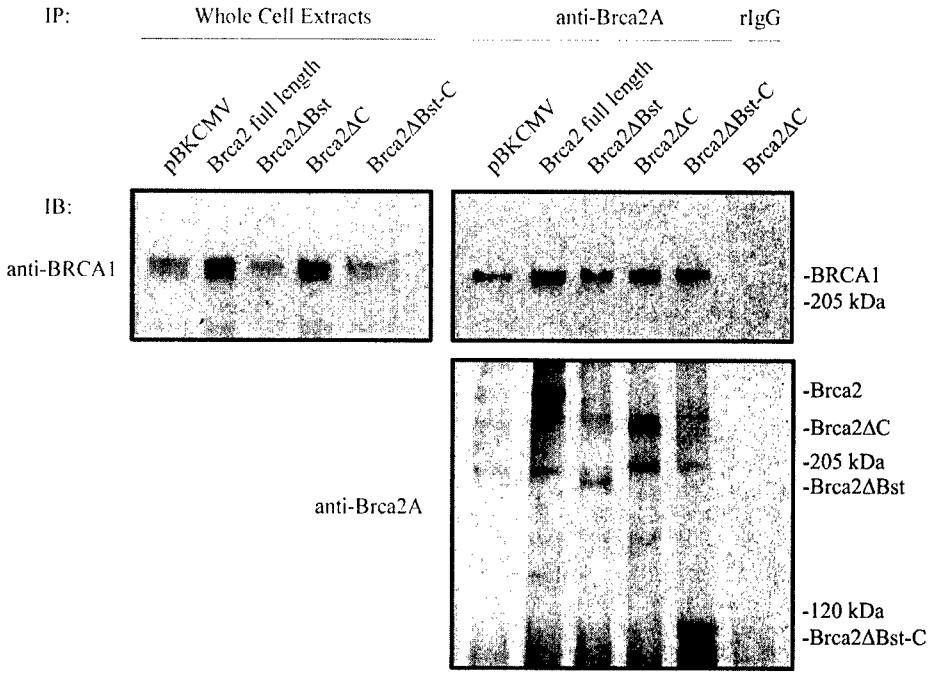

FIG. 6. **The amino terminus of Brca2 is sufficient for interaction with BRCA1.** 293T cells were transfected with 12.5 μg of *pBKCMV*, 12.5 μg of full-length *Brca2*, 2 μg of *Brca2ΔBst*, 12.5 μg of *Brca2ΔC*, or 2 μg of *Brca2ΔBst-C*. All transfections included 12.5 μg of a *BRCA1* expression plasmid and sufficient *pBKCMV* to bring the total DNA to 25 μg per transfection. Whole cell extracts (*left panel*) or anti-Brca2A immunoprecipitates (*right panels*) from transfected cells were immunoblotted (*IB*) with anti-BRCA1 MS110 (*top panels*) or anti-Brca2A (*bottom panel*). *IP*, immunoprecipitated.

quences mediating this interaction have been evolutionarily conserved.

The Amino Terminus of Murine Brca2 Is Sufficient for Nuclear Localization—Human BRCA2 has been shown to contain three nuclear localization signals at its extreme carboxyl terminus (3, 4). Accordingly, human *BRCA2* alleles that harbor truncating mutations anywhere within the gene result in BRCA2 polypeptides that localize to the cytoplasm and that are therefore presumed to be nonfunctional (3). If the signals directing nuclear localization in murine Brca2 are similarly positioned, even small carboxyl-terminal truncations in murine Brca2 should result in a cytoplasmic gene product. These findings predict that truncating mutations anywhere within *Brca2* would result in equally severe phenotypes. In contrast to this prediction, the viability of mice bearing truncations in *Brca2* directly correlates with the length of the resulting protein, in that amino-terminal truncations have more severe phenotypes than carboxyl-terminal truncations. We reasoned that it was more likely that murine and human BRCA2 would have conserved functions but different nuclear localization signal locations than have conserved nuclear localization signal locations and different functions. We tested this hypothesis using immunofluorescence to analyze the subcellular location of Brca2 polypeptides in 293T cells transiently transfected with the above murine *Brca2* deletion constructs.

As expected, exogenously expressed full-length murine Brca2 was observed to localize exclusively to the nucleus, as demonstrated by co-fluorescence with ECFP-Nuc (CLONTECH), a control for nuclear localization (Fig. 7). Similarly, Brca2ΔBst was also shown to localize to the nucleus of transfected cells, indicating that exon 11 is not required for the nuclear localization of murine Brca2. Surprisingly, Brca2ΔBst-C was also shown to localize to the nucleus despite its deletion of more than three-fourths of the full-length protein, including the carboxyl terminus. Brca2ΔBst-C encodes a polypeptide with a predicted molecular mass of 82 kDa that is significantly greater than the 65-kDa molecular mass cutoff for passive diffusion through nuclear pores (39). As such, the nuclear localization of Brca2ΔBst-C cannot be explained by simple diffusion. Moreover, no fluorescent signal was detected in control cells transfected with the empty vector, indicating that the apparent localization of Brca2ΔBst-C is not the result of antibody cross-reactivity with endogenous human BRCA2. These findings suggest that the amino terminus of murine Brca2 is sufficient to direct the nuclear localization of this protein.

DISCUSSION

We have demonstrated that the murine Brca2 protein is similar to human BRCA2 with regard to its nuclear localization, cell cycle regulation, binding to Brca1, and binding to Rad51. In addition, we have defined further the domains of Brca2 that are required for its interaction with RAD51 and BRCA1. Finally, despite low overall homologies between the murine and human orthologs of BRCA2 and BRCA1, we have demonstrated that murine Brca2 is capable of stably interacting with human BRCA1 *in vivo*. This indicates that the interaction between Brca1 and Brca2 has been conserved evolutionarily and suggests that this interaction is functionally important. In aggregate, our data are consistent with the hypothesis that murine and human BRCA2 have largely equivalent functions.

One notable difference that we observed between murine and human BRCA2 was the finding that the amino terminus of murine Brca2 appears to be sufficient for its nuclear localization. In contrast, analysis of human BRCA2-GFP fusion proteins has demonstrated that truncating even 155 residues from the carboxyl terminus of BRCA2 completely abrogates its nuclear localization (3). Similarly, endogenous BRCA2 in CAPAN-1 cells, which lacks the carboxyl-terminal third of BRCA2, has been shown to localize to the cytoplasm by biochemical fractionation (3). As such, we believe that our findings reflect differences in the placement of nuclear localization sequences within human and murine Brca2. A potential caveat to this interpretation is that our localization studies were performed on Brca2 polypeptides expressed ectopically in human 293T cells rather than in murine cells. However, our conclusions are supported by the finding that a targeted deletion of the final 566 coding nucleotides of *Brca2* results in a polypeptide that localizes to the nucleus in murine cells (40). Paradoxically, the difference in positioning of nuclear localization signals that we have identified between murine and human BRCA2 strengthens the hypothesis that murine and human BRCA2 are functionally equivalent. If, as for human BRCA2, truncation at the

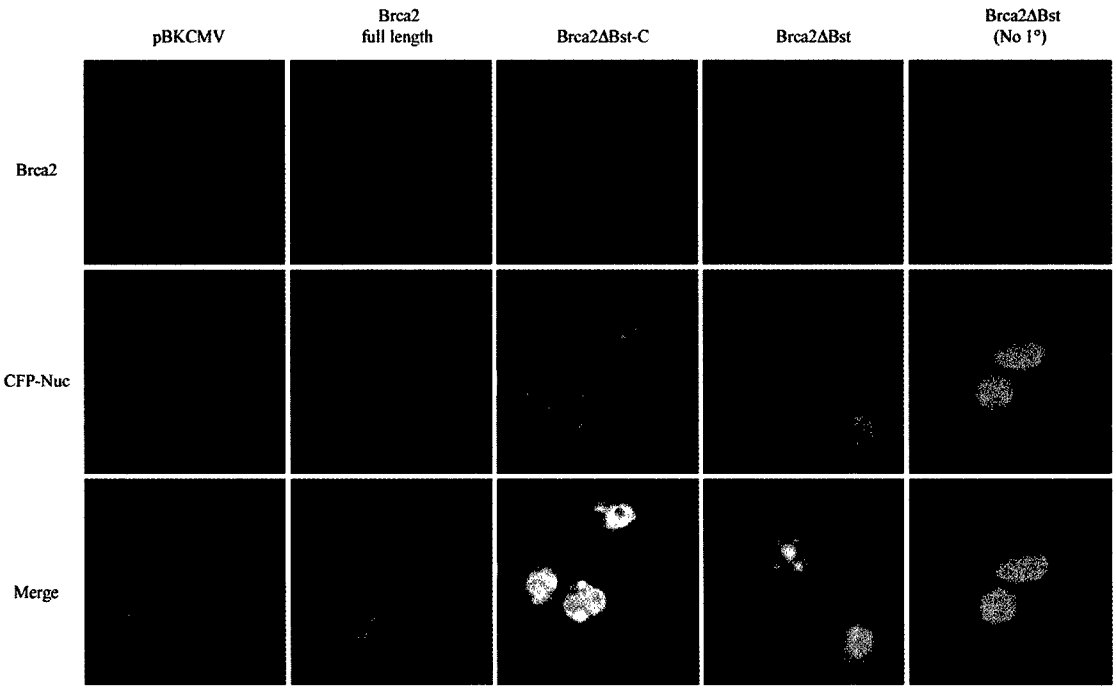

FIG. 7. **The amino terminus of murine Brca2 is sufficient for nuclear localization.** 293T cells were transiently transfected with 4.5 μg of the indicated *Brca2* deletion constructs and 0.5 μg of *pECFP-Nuc*. Cells were analyzed for Brca2 subcellular localization by indirect immunofluorescence with anti-Brca2A and co-fluorescence with CFP-Nuc.

extreme carboxyl terminus of murine Brca2 resulted in cytoplasmic localization, we would have been forced to conclude that the more severe phenotype of mice bearing amino-terminal compared with carboxyl-terminal truncating *Brca2* mutations reflected differences in the functions of murine and human BRCA2. Our demonstration that carboxyl-terminal truncations of murine Brca2 remain nuclear resolves this dilemma.

Mice homozygous for truncating mutations within exon 11 exhibit reduced embryonic survival, spontaneous tumorogenesis, genomic instability, and reduced Rad51 nuclear foci formation following DNA damage (27–30). Our finding that Brca2 polypeptides lacking exon 11 are incapable of co-immunoprecipitating RAD51 constitutes the first biochemical evidence that exon 11 is required for the interaction of Brca2 with RAD51. This, along with our observation that Brca2 mutants lacking the carboxyl terminus retain their capacity to bind to RAD51, is consistent with yeast two-hybrid studies of the human BRCA2-RAD51 interaction and suggests that exon 11 is the principal domain of murine Brca2 required for binding to RAD51. Nevertheless, given that interactions of the carboxyl terminus of murine Brca2 with Rad51 have been detected by two different approaches, we favor the possibility that this region may contribute to the interaction of Brca2 with Rad51. The targeted, in-frame deletion of exon 11 in mice would permit a more accurate assessment of the role of the BRC repeats in binding to Rad51 and of the impact that this interaction has on DNA repair and tumor susceptibility.

The multiple similarities between human and murine Brca2 that we have demonstrated in this report shed new light on observations made in mice bearing targeted mutations in *Brca2*. Mice lacking the small carboxyl-terminal domain of Brca2 shown to interact with Rad51 have not been reported to develop tumors, although cells from such mice exhibit premature senescence and decreased efficiency in homology-based DNA repair (31, 40). Our data predict that mice bearing carboxyl-terminal deletions would have at most only slightly impaired binding of Brca2 to Rad51. This, in turn, may explain the more modest phenotype of mice bearing such mutations. In support of this hypothesis, Moynahan and colleagues (40) have shown recently that the amount of Brca2 bound to Rad51 in murine embryonic stem cells is unaffected by deletion of the carboxyl-terminal domain of *Brca2*. As such, the premature senescence and decreased DNA repair phenotypes observed in these mice may be due either to an uncharacterized defect in the Rad51 pathway or to the disruption of interactions with other proteins involved in homology-based DNA repair.

Finally, our finding that the amino terminus of Brca2 is sufficient to interact with BRCA1 suggests that Brca2-Brca1 complexes may be maintained in all *Brca2* knockout mouse models generated to date; however, whether such Brca2-Brca1 complexes retain their function is unknown. Both *Brca2* and *Brca1* mutant cells have defects in Rad51 focus formation following DNA damage (36, 41). We have recently demonstrated that murine Brca1, like murine Brca2, localizes to nuclear foci (36). As peptides bearing consensus BRC repeat sequences can inhibit the polymerization of RAD51 onto DNA substrates *in vitro* (42), these data collectively suggest a role for Brca2, and potentially Brca1, in recruiting or preparing Rad51 for subsequent recombination events at sites of DNA damage. Nevertheless, it has yet to be demonstrated how Brca2 and Brca1 orchestrate Rad51 nuclear focus formation following DNA damage, and it has not been determined whether the disrupted regulation of Rad51 nuclear focus formation is ultimately responsible for the malignant transformation of *Brca1* and *Brca2* mutant cells. Such studies should enhance our understanding of the mechanism by which *Brca1* and *Brca2* gene products suppress tumor formation.

Acknowledgments—We thank Sherry M. Wang and Edward J. Gunther for provision of murine *Brca2* clones for use in the isolation of the *Brca2* cDNA. We also thank James Sanzo, Neelima Shah, and Irina Chernysh of the University of Pennsylvania Biomedical Imaging Core Laboratory for assistance with confocal microscopy and Hank Pletcher of the University of Pennsylvania Flow Cytometry and Cell Sorting Shared Resource Facility for assistance with FACS analysis.

REFERENCES

1. Ford, D., Easton, D. F., Stratton, M., Narod, S., Goldgar, D., Bishop, D. T., Weber, B., Lenoir, G., Chang-Claude, J., Sobol, H., Teare, M. D., Struewing, J., Arason, A., Scherneck, S., Peto, J., Rebbeck, T. R., Tonin, P., Neuhausen, S., Barkardottir, R., Eyfjord, J., Lynch, H., Ponder, B. A., Gayther, S. A., Zelada-Hedman, M., *et al.* (1998) *Am. J. Hum. Gen.* **62,** 676–689
2. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. (1995) *Nature* **378,** 789–792
3. Spain, B. H., Larson, C. J., Shihabuddin, L. S., Gage, F. H., and Verma, I. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13920–13925
4. Yano, K., Morotomi, K., Saito, H., Kato, M., Matsuo, F., and Miki, Y. (2000) *Bioch. Biophys. Res. Commun.* **270,** 171–175
5. Vaughn, J. P., Cirisano, F. D., Huper, G., Berchuck, A., Futreal, P. A., Marks, J. R., and Iglehart, J. D. (1996) *Cancer Res.* **56,** 4590–4594
6. Bertwistle, D., Swift, S., Marston, N. J., Jackson, L. E., Crossland, S., Crompton, M. R., Marshall, C. J., and Ashworth, A. (1997) *Cancer Res.* **57,** 5485–5488
7. Vaughn, J. P., Davis, P. L., Jarboe, M. D., Huper, G., Evans, A. C., Wiseman, R. W., Berchuck, A., Iglehart, J. D., Futreal, P. A., and Marks, J. R. (1996) *Cell Growth Differ.* **7,** 711–715
8. Chen, Y., Farmer, A. A., Chen, C. F., Jones, D. C., Chen, P. L., and Lee, W. H. (1996) *Cancer Res.* **56,** 3168–3172
9. Ruffner, H., and Verma, I. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7138–7143
10. Gudas, J. M., Li, T., Nguyen, H., Jensen, D., Rauscher, F. J., III, and Cowan, K. H. (1996) *Cell Growth Differ.* **7,** 717–723
11. Chen, F., Nastasi, A., Shen, Z., Brenneman, M., Crissman, H., and Chen, D. J. (1997) *Mutat. Res.* **384,** 205–211
12. Chen, P. L., Chen, C. F., Chen, Y., Xiao, J., Sharp, Z. D., and Lee, W. H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5287–5292
13. Marmorstein, L. Y., Ouchi, T., and Aaronson, S. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13869–13874
14. Wong, A. K. C., Pero, R., Ormonde, P. A., Tavtigian, S. V., and Bartel, P. L. (1997) *J. Biol. Chem.* **272,** 31941–31944
15. Katagiri, T., Saito, H., Shinohara, A., Ogawa, H., Kamada, N., Nakamura, Y., and Miki, Y. (1998) *Genes Chromosomes Cancer* **21,** 217–222
16. Chen, J. J., Silver, D. P., Walpita, D., Cantor, S. B., Gazdar, A. F., Tomlinson, G., Couch, F. J., Weber, B. L., Ashley, T., Livington, D. M., and Scully, R. (1998) *Mol. Cell* **2,** 317–328
17. Bork, P., Blomberg, N., and Nilges, M. (1996) *Nat. Genet.* **13,** 22–23
18. Bignell, G., Micklem, G., Stratton, M. R., Asworth, A., and Wooster, R. (1997) *Hum. Mol. Genet.* **6,** 53–58
19. Sharan, S. K., Morimatsu, M., Albrecht, U., Lim, D. S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., and Bradley, A. (1997) *Nature* **386,** 804–810
20. Suzuki, A., de la Pompa, J. L., Hakem, R., Elia, A., Yoshida, R., Rong, M., Nishina, H., Chuang, T., Wakeham, A., Itie, A., Koo, W., Billia, P., Ho, A., Fukumoto, M., Hui, C. C., and Mak, T. W. (1997) *Genes Dev.* **11,** 1242–1252
21. Ludwig, T., Chapman, D. L., Papioannou, V. E., and Efstradiatis, A. (1997) *Genes Dev.* **11,** 1226–1241
22. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6236–6240
23. Gowen, L. C., Johnson, B. L., Latour, A. M., Sulik, K. K., and Koller, B. H. (1996) *Nat. Genet.* **12,** 191–194
24. Hakem, R., de la Pompa, J. L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., Firpo, E., Hui, C. C., Roberts, J., Rossant, J., and Mak, T. W. (1996) *Cell* **85,** 1009–1023
25. Lim, D. S., and Hasty, P. (1996) *Mol. Cell. Biol.* **16,** 7133–7143
26. Hakem, R., de la Pompa, J. L., Elia, A., Potter, J., and Mak, T. W. (1997) *Nat. Genet.* **16,** 298–302
27. Connor, F., Bertwistle, D., Mee, P. J., Ross, G. M., Swift, S., Grigorieva, E., Tybulewicz, V. L. J., and Ashworth, A. (1997) *Nat. Genet.* **17,** 423–430
28. Friedman, L. S., Thistlewaite, F. C., Patel, K. J., Yu, V. P. C. C., Lee, H., Venkitaraman, A. R., Abel, K. J., Carlton, M. B. L., Hunter, S. M., Coledge, W. H., Evans, M. J., and Ponder, B. A. J. (1998) *Cancer Res.* **58,** 1338–1343
29. Patel, K., Yu, V. P. C. C., Lee, H., Corcoran, A., Thistlewaite, F. C., Evans, M. J., Colledge, W. H., Friedman, L. S., Ponder, B. A. J., and Venkitaraman, A. R. (1998) *Mol. Cell* **1,** 347–357
30. Yu, V. P., Koehler, M., Steinlein, C., Schmid, M., Hanakahi, L. A., van Gool, A. J., West, S. C., and Venkitaraman, A. R. (2000) *Genes Dev.* **14,** 1400–1406
31. Morimatsu, M., Donoho, G., and Hasty, P. (1998) *Cancer Res.* **58,** 3441–3447
32. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, 1st Ed., pp. 313–318, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (2000) *Current Protocols in Molecular Biology*, 2nd Ed., pp. 9.1.4–9.1.6, John Wiley & Sons, Inc., New York
34. Wilson, C. A., Ramos, L., Villasenor, M. R., Anders, K. H., Press, M. F., Clarke, K., Karlan, B., Chen, J. J., Scully, R., Livingston, D., Zuch, R. H., Kanter, M. H., Cohen, S., Calzone, F. J., and Slamon, D. J. (1999) *Nat. Genet.* **21,** 236–240
35. Rajan, J. V., Wang, M., Marquis, S. T., and Chodosh, L. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13078–13083
36. Huber, L. J., Yang, T. W., Sarkisian, C. J., Master, S. R., Deng, C. X., and Chodosh, L. A. (2001) *Mol. Cell. Biol.* **21,** 4005–4015
37. Baer, R., and Lee, W. H. (1998) *J. Mammary Gland Biol. Neoplasia* **3,** 403–412
38. Mizuta, R., LaSalle, J. M., Cheng, H.-L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N. A., LaLande, M., and Alt, F. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6927–6932
39. Gorlich, D., and Mattaj, I. W. (1996) *Science* **271,** 1513–1518
40. Moynahan, M. E., Pierce, A. J., and Jasin, M. (2001) *Mol. Cell* **7,** 263–272
41. Bhattacharyya, A., Ear, U. S., Koller, B. H., Weichselbaum, R. R., and Bishop, D. K. (2000) *J. Biol. Chem.* **275,** 23899–23903
42. Davies, A. A., Masson, J.-Y., McIlwraith, M. J., Stasiak, A. Z., Stasiak, A., Venkitaraman, A. R., and West, S. C. (2001) *Mol. Cell* **7,** 273–282